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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Tanimoto, et al.  
Appl. No. : 09/514,999  
Filed : February 29, 2000  
For : METHOD OF  
MANUFACTURING  
POLYAMINE COMPOSITION  
Examiner : I. Marx  
Group Art Unit : 1651

DECLARATION UNDER RULE 132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

I, YOSHIHIRO TANIMOTO, do hereby declare and say:

1 I am a co-inventor of the above-referenced application.

2 The yeast somatic components were subjected to the decomposition step for 15-18 hours as described in Examples 1-3 of the present specification. This was because we simply left the reaction materials overnight for convenience as is common practice for enzyme-related reactions. This was not because we thought that a time period of 15-18 hours was essential. If we set and left the experiments at 5 p.m. and came back at 9 a.m. the next day for evaluation, it would be 16 hours. We never state in the specification as originally filed that a time period of 15-18 hours was essential. On the contrary, we specifically state in the specification as originally filed that the reaction can be accomplished in 0.1 to 24 hours on page 5, lines 8 and 19.

3 The following experiments using the methods taught in the present application were performed by me or under my direct supervision or control to show that a time period of 15-18 hours is not essential and a time period of 0.1-24 hours as originally stated is sufficient to achieve nearly as high a yield as in Examples 1-3 of the present specification.

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4 Preparation of Polyamine Solution: Yeast ribonucleic acid (Kirin Beer Co.) was employed as the yeast somatic components. The yeast ribonucleic acid was dissolved in a 10 mM acetic acid buffer solution (pH 5.0) at a concentration of 2% (w/vol). A nuclease (NUCLEASE AMANO, Amano Enzyme K.K.) was added to the yeast ribonucleic acid solution at a concentration of 2 mg/ml and reaction was conducted at 37°C; the reaction supernatant was recovered at the start of the reaction (time zero), and at 0.1, 1, 3, and 18 hours after the commencement of the reaction. The recovered reaction supernatant was adjusted to a pH of 1.5 by adding 4N hydrochloric acid, and after remaining in an ice water bath for 30 minutes, centrifugal separation was performed (3000 rpm, 4°C, 15 minutes) and a *pellet* was precipitated. The recovered supernatant was a polyamine solution.

5 Evaluation of Polyamine Concentration: This polyamine solution can be concentrated using the ion-exchange resin process described in Example 1 described in the present application. The amount of polyamine present in the recovered polyamine solution was measured and the polyamine yield was evaluated. That is, 300  $\mu$ l of 3N perchloric acid was added to 600  $\mu$ l of polyamine solution and this was placed in an ice water bath for 20 minutes, subjected to centrifugation (15000 rpm, 4°C, 15 minutes), and the supernatant was recovered and used for analysis. The polyamine content was analyzed by high-performance liquid chromatography using *o*-phthalaldehyde.

6 Results: Relative values for the amount of polyamine present at the specified intervals in the polyamine solution obtained by nuclease digest are depicted in the table and figure shown below. The amount of polyamine present in the polyamine solution at reaction time zero was set equal to 100%. The results show that even after the reaction had progressed only 0.1 hours, the polyamine yield of the polyamine solution approximately doubled in comparison with the yield when no nuclease digestion was performed. When the reaction proceeded for an hour or more, the yield was 5-6 times that obtained without nuclease digestion. These results show that under these digestion conditions, the polyamine yield in the polyamine solution at least doubles when the nuclease digest period is 0.1 hours or more.

7 Table

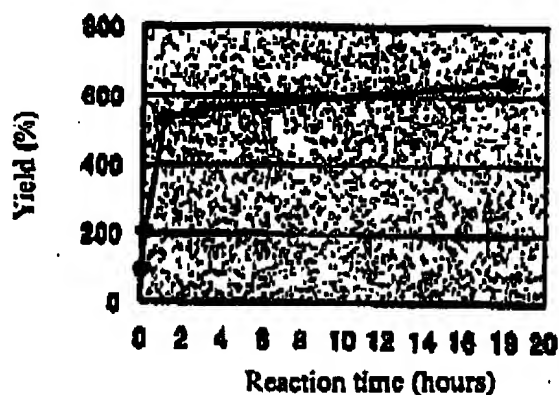
Reaction Time (hours)	Polyamine Yield (%)
0	100

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0.1	206
1	536
3	560
18	638

8 Figure



9 I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Dated: 07.01.2004

By: Yoshihiro Tanimoto  
Yoshihiro Tanimoto

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